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PROTEIN KINASE C ACTIVITY IN COMPENSATORY KIDNEY GROWTH

Carlos Caramelo, Phoebe Tsai,
Koji Okada, and Robert W. Schrier

Department of Medicine
University of Colorado School of Medicine
Denver, Colorado 80262

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SUMMARY: Very little information exists about the intracellular mechanisms mediating remnant kidney hypertrophy after reduction of renal mass. The present experiments demonstrate that the activity of a key enzyme in cellular proliferation, protein kinase C, is enhanced in the kidney during post-uninephrectomy hypertrophy. As additional evidence of "in vivo" enzyme activation, the enzyme activity migrates from the cytosolic to the particulate fraction of the cells and the number of sites of phorbol dibutyrate binding is enhanced in kidney membranes 24 hr after contralateral nephrectomy. The stimulation of the enzyme is higher than the increase in kidney weight, although both phenomena are linearly related.

Mammalian kidneys respond to nephron loss by undergoing compensatory growth (1). In adult animals, about 80% of kidney growth following uninephrectomy can be ascribed to cell hypertrophy, rather than an increase in cell number (1).

In the last two decades the interest in renal compensatory growth has increased because of the general use of renal transplantation and by the putative role of individual nephron hypertrophy and hypermetabolism in the progression of chronic renal disease (2). However, little is known about the actual mechanisms involved in the compensatory growth and no conclusive evidence is available about the intracellular mediators of this phenomenon. Indirect evidence from renal cell lines in culture have implicated some factors in the culture media in the process of cell growth, including epidermal growth factor (3,4), insulin (3), platelet-derived growth factor (1,3), NaCl concentration (1,5) and hormones such as arginine vasopressin (1) and angiotensin (1). Other studies disclosed the important role of ion fluxes including Na^+, H^+, K^+, and Ca^{2+} in the initiation of cell growth (1,5,6). For example, the activity of the Na^+:H^+ antiporter appears to be consistently increased "in vivo" after a reduction of renal cell mass (5-7).

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In search of a common intracellular process mediating the above-mentioned growth factors, the activation of the enzyme protein kinase C may be involved. Specifically, protein kinase C has been shown to be activated by growth factors (8,9). Protein kinase C, as well as growth factors, stimulates the Na^+:H^+ antiporter and Na^+ influx in different cellular types (8). The protein kinase C activation by tumor promoters such as phorbol esters or diacylglycerol is well known to induce cell growth (10,11). Increased activity of protein kinase C has been also reported in epithelial cells during proliferation (12).

Structurally, protein kinase C possesses a hydrophobic regulatory domain and a catalytic domain which induces ATP-phosphorylation of a number of intracellular proteins (8-10). The enzyme has been identified in two cellular fractions, cytosolic or soluble and membrane-bound or particulate (9,10,13). Activation of protein kinase C by several factors increases the relative ratio of the particulate to soluble fraction by a mechanism which probably involves Ca^{2+} (9,10,14). The enzyme has a complex activation system, which includes calcium, phosphatydylserine, and diacylglycerol (DAG), a product of phospholipase C action on membrane phospholipids. DAG reduces the enzyme's Km for Ca^{2+} to values close to that normally existing in the cytosol (10^{-7} M) (9,10,15). The activating effects of DAG can be mimicked by phorbol esters (16) which also specifically bind to protein kinase C (17).

The present experiments were designed to determine whether protein kinase C activity is stimulated "in vivo" during compensatory kidney growth. Unilateral nephrectomy was chosen as the most reproducible method of renal mass reduction. Twenty-four hours after removal of the first kidney the experimental kidney was removed and studied since compensatory growth has been shown to be significantly activated by this time (1).

MATERIALS AND METHODS

Nephrectomy and Preparation of Subcellular Fractions: Sprague-Dawley rats weighing between 210 and 230 g were used for the experiments. Under light pentobarbital anesthesia (3.23 mg/100 g weight), uninephrectomy was performed and the kidney cortex was dissected on an ice-cold plate. After total kidney and cortex weights were obtained, cortex was homogenized in a medium with 0.25 M sucrose, 20 mM Tris, 5 mM dithiothreitol (DTT), 2 mM EGTA, 2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 100 µg/ml leupeptin, as described by Uchida and Filburn (18), in a glass homogenizer with a Teflon pestle, followed by three bursts of Polytron (15 sec each, speed 7). The homogenate was centrifugated for 10 min at 10,000xg and the supernatant of this centrifugation was again centrifuged at 104,000xg, 60 min (Beckman L8 60M). The ultracentrifugation supernatant (soluble fraction) was adjusted to 1 µM with cAMP and applied to a DEAE-Sephacel column (12x30 mm) and sequentially eluted with 15 ml of cAMP-containing homogenization buffer minus sucrose and a 0.025 to 0.25 M NaCl gradient in 20 mM Tris, pH 7.5, with 5 mM DTT and 0.1 mM PMSF. The ultracentrifugation pellet was redissolved in 1 ml of homogenizing buffer containing 0.1% Tryton X100 and submitted to a second

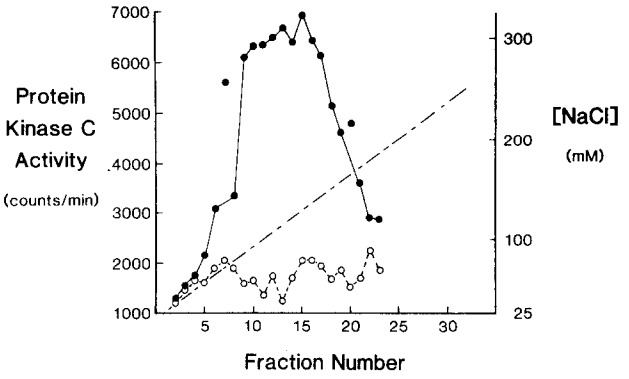

Fig. 1. Protein kinase C activity of the DEAE-sephacel column NaCl gradient elution fraction: a) phosphatidyl-serine, diolein and Ca^{2+} activated protein kinase C activity (closed circles); b) basal protein kinase C activity (open circles); and c) NaCl gradient (dotted/dashed lines).

ultracentrifugation (104,000xg, 60 min); the second supernatant, corresponding to the particulate fraction, was purified with the same method as the soluble fraction. All of the above procedures were carried out at 4°C.

Enzyme Assay: Aliquots of all the samples obtained from the columns were assayed for protein kinase C activity by triplicate (Fig. 1), by a slight modification of the method used by Uchida and Filburn (13). In brief, the reaction mixture contained 25 mM Tris, pH 7.5, 20 µg of histone III-S, 5 mM $MgCl_2$, 20 µM ATP, 1-2x10^{-6} cpm [^{32}P]ATP, 100 µM EGTA, ±50 µg/ml of phosphatydylserine, 0.5 µg/ml of diolein and 2 mM free Ca^{2+}, in a total volume of 100 µl. Reactions were initiated by addition of 20 µl of enzyme and terminated after 1 min, 30°C incubation, by the addition of 10% trichloroacetic acid (TCA), 5 mM NaH_2PO_4, 2 mM ATP, followed by 100 µl of 0.5 BSA. Precipitated protein was sedimented by centrifugation, and washed twice with 10% TCA, 5 mM NaH_2PO_4. The protein pellet was counted in a scintillation spectrometer.

Phorbol Dibutyrate (PDB) Binding: We used a modification of the methods of Wolf et al (19) and Moruzzi et al (20). Fifty µl of resuspended membranes after 104,000xg centrifugation were added to 50 µl of a reaction mixture containing 10 mM Tris Cl, pH 7.4, 2 mM dithiothreitol, 10 mM $MgCl_2$, 100 µM $CaCl_2$, 10 µg bovine serum albumin, and 40 µM ^{3}H-PDB for 10 min at 25°C in the absence or presence of different concentrations of radioinert PDB; 10^{-6} M PDB was used for the determination of non-specific binding. Bound and free ^{3}H-PDB were separated by adding gamma-globulin-polyethylengycol and centrifugating (Eppendorf Microcentrifuge). Experiments were done before and after overnight dialysis of the membranes (5961 FA membrane, Bethesda Research Laboratories, Gaithesburg, MD), to eliminate dialyzable PDB binding inhibitors that may be present in non-purified tissue extracts. Results of the competition curves were calculated by the EBDA-LIGAND program (Elsevier Science Publ., Holland). In all cases, aliquots of the samples were tested for protein concentration by the method of Lowry (21).

Statistical Analysis: Double way variance analysis, the Student's "t" test and linear regression were used for statistical comparison.

RESULTS

After 24 hr, the weight of the second kidney (remnant kidney) increased 10.29±3.14% relative to the contralateral kidney which had been removed initially ($p<.05$). There was an increase in total and specific protein kinase

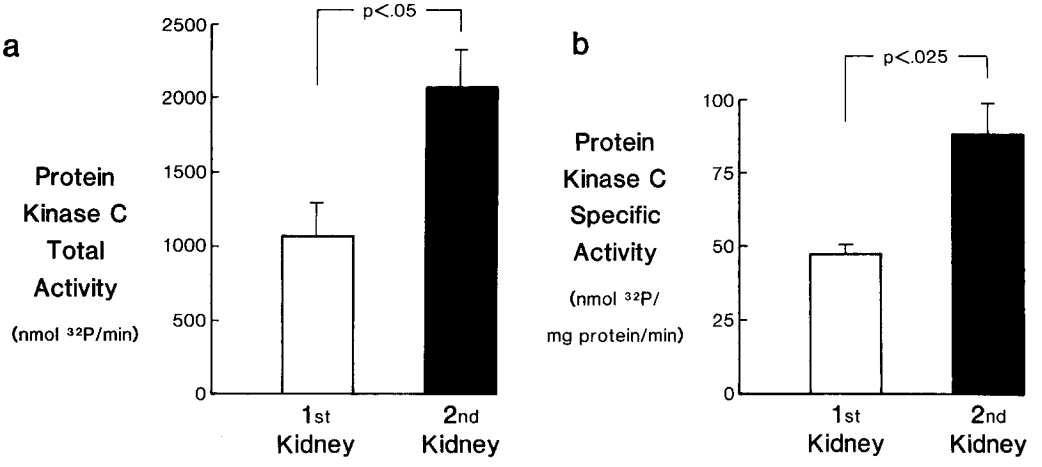

Fig. 2a. Protein kinase C activity in control and remnant kidney (n=5 rats, mean ± SEM). Values were normalized to 1 gram of cortex tissue.

Fig. 2b. Protein kinase C specific activity in control and remnant kidney (n=5 rats, mean ± SEM).

C activity in the hypertrophied as compared to the contralateral kidney which had been removed 24 hr earlier (Figures 2a and 2b). Basal protein kinase C activity was in the same range found by other authors (13). In addition, the percentage of the enzyme isolated from the particulate fraction was significantly increased in the remnant kidneys (Figure 3). Parallel studies in sham-operated rats (n=3) demonstrated neither differences in protein kinase C activities nor in the particulate/soluble distribution between both kidneys (data not shown). A positive correlation (p<.01) was found between the increase in protein kinase C activity and the increase in the experimental

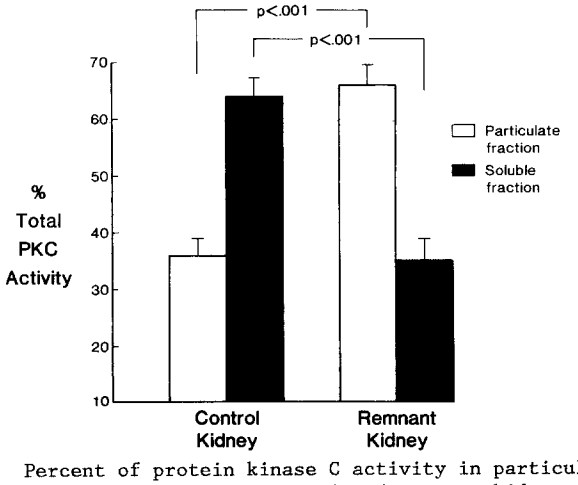

Fig. 3. Percent of protein kinase C activity in particulate versus soluble fraction in control and remnant kidneys (n=5 rats, mean ± SEM).

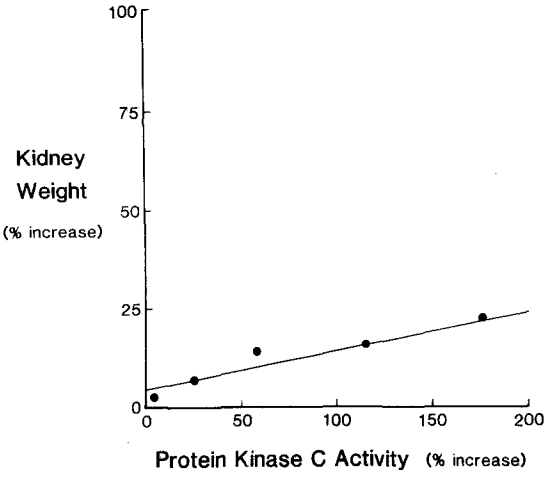

Fig. 4. Linear regression of percent increase in total protein kinase C activity against percent increase in kidney weight ($r=0.902$, $p<.01$). $y=3.48+0.0918x$. The greater percent increase in protein kinase C activity as compared with percent increase in kidney weight determines the slope of the curve to significantly deviate from that corresponding to a 1:1 ratio.

kidney weight (Figure 4). The results of phorbol ester binding experiments are shown in Table 1. The Hill coefficient was between 0.94 and 0.99 in all the groups, suggesting the presence of one binding site. The number of binding sites is significantly increased in the remnant kidney as compared to the nephrectomy kidney. Treatment by dialysis induced a nearly significant decrease in Kd that suggests that a competitive dialyzable inhibitor of phorbol ester binding may be present in crude kidney extracts. Figure 5 illustrates one PDB binding experiment. Additional binding experiments were done in rat brain (Kd 86.7 nM, Bmax 6.78×10^{-12} pg/mg prot) and spleen (Kd 98.0 nM, Bmax 9.96×10^{-12} pg/mg prot), as a control of the results from kidneys.

DISCUSSION

The present results indicate "in vivo" activation of protein kinase C in the remnant kidney of uninephrectomized rats, as recently suggested in

Table 1. Binding of ^{3}H-PDB to kidney membranes

	First Kidney		Second Kidney	
	No Dialysis	Dialysis	No Dialysis	Dialysis
Kd (nM)	69.51±11.89	44.7±10.59	68.35±9.90	56.53±6.21
Bmax (pg/mg prot)	1.0±0.34	1.14±0.43	2.73±0.75*	2.81±0.77*

Results are the mean±SEM of 5 triplicate experiments.

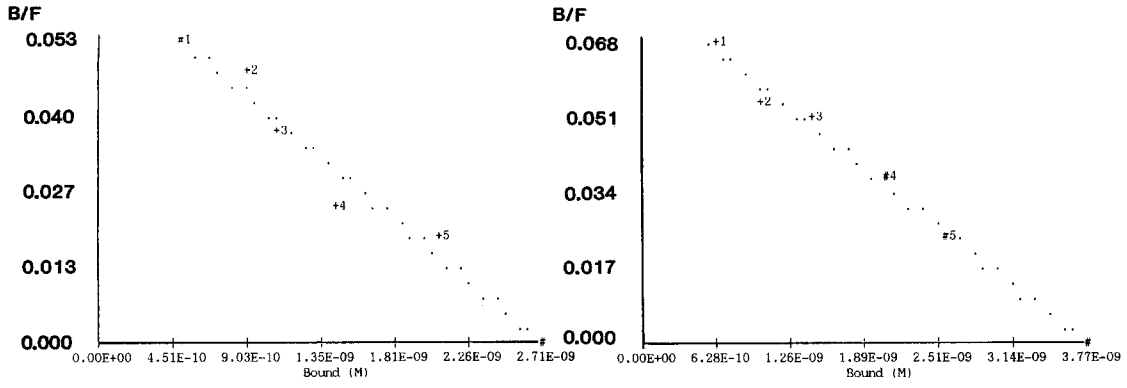

Fig. 5. Binding of ^{3}H-PDB to binding membranes before (left) and after (right) uninephrectomy. Figures are obtained from the EBDA-LIGAND program. Each point corresponds to triplicate determinations.

preliminary results from another laboratory (22). The increase in both total activity of the enzyme as well as the translocation from the soluble to the particulate fraction provide evidence for such activation. The kinetic data from the PDB binding studies suggest that the observed activation may be due to changes in the number of membrane-attached units of the enzyme. Also, the present results demonstrate that, at least 24 hr after the nephrectomy, there is no down-regulation of protein kinase C, as has been shown "in vitro" after prolonged activation by phorbol esters (10).

The activation of protein kinase C may provide some explanations for several of the following phenomena which accompany compensatory hypertrophy including: a) increased Na^+:H^+ transporter (1,5-7); b) increased cellular Na^+ uptake and subsequent stimulation of Na^+/K^+-ATPase, as a corollary of Na^+:H^+ antiporter stimulation (1,23); c) increased amino acid transport, which has been detected in the kidney undergoing hypertrophy (24) and can be stimulated by protein kinase C (25); d) increased activity of ornithine decarboxylase, the regulatory enzyme of polyamine's synthesis (1), which has been recently shown to be induced by protein kinase C-dependent phosphorylation (26); interestingly, polyamines, which are increased in the remnant kidney (1), have been shown to inhibit protein kinase C activity (10,20); e) increased Ca accumulation in endoplasmic reticulum vesicles (27), a phenomenon that can be potentiated by protein phosphorylation (28); and f) increased prostaglandin synthesis, which has been proposed to occur in the remnant kidney (29) and can be stimulated by protein kinase C activation (30).

Potentially, protein kinase C activation may provide new directions for unexplained facts of compensatory hypertrophy, e.g. the triggering mechanism of hypertrophy and hyperplasia. In spite of the information obtained in the present experiments, a time course of the enzyme activation from the earliest

moments after the nephrectomy, and studies of the enzyme activity in different tubular and glomerular fractions, as well as the observation of kidney hypertrophy after inhibiting protein kinase C, need to be performed to ascertain further the actual importance of protein kinase C activation in the regulation of kidney growth.

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